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**Low oxygen affects photophysiology and the level of expression of two carbon metabolism genes in the seagrass *Zostera muelleri*.**

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## ABSTRACT

Seagrasses are a diverse group of angiosperms that evolved to live in shallow coastal waters, an environment regularly subjected to changes in oxygen, carbon dioxide and irradiance. *Zostera muelleri* is the dominant species in south-eastern Australia, and is critical for healthy coastal ecosystems. Despite its ecological importance, little is known about the pathways of carbon fixation in *Z. muelleri* and their regulation in response to environmental changes. In this study, the response of *Z. muelleri* exposed to control and very low oxygen conditions was investigated by using (i) oxygen microsensors combined with a custom-made flow chamber to measure changes in photosynthesis and respiration, and (ii) Reverse Transcription quantitative real-time PCR (RT-qPCR) to measure changes in expression levels of key genes involved in C<sub>4</sub> metabolism. We found that very low levels of oxygen (i) altered the photophysiology of *Z. muelleri*, a characteristic of C<sub>3</sub> mechanism of carbon assimilation, and (ii) decreased the

expression levels of phosphoenolpyruvate carboxylase (PEPC) and carbonic anhydrase (CA). These molecular-physiological results suggest that regulation of the photophysiology of *Z. muelleri* might involve a close integration between the C<sub>3</sub> and C<sub>4</sub>, or other CO<sub>2</sub> concentrating mechanisms metabolic pathways. Overall, this study highlights that the photophysiological response of *Z. muelleri* to changing oxygen in water is capable of rapid acclimation and the dynamic modulation of pathways should be considered when assessing seagrass primary production.

## **INTRODUCTION**

Seagrasses are a diverse group of monocotyledonous angiosperms that evolved to live in the marine environment during the Cretaceous period, approximately 100 million years ago (Larkum and den Hartog 1989). There are approximately 72 seagrass species in 12 genera worldwide (Short et al. 2011) playing an important role in coastal ecosystems (Costanza et al. 1997). Indeed, highly productive seagrass ecosystems provide food and shelter for commercially important fish (Beck et al. 2001) with temperate seagrass meadows in southern Australia estimated to supply onshore fisheries valued at \$A 230 000 ha<sup>-1</sup> y<sup>-1</sup> (Blandon and Zu Ermgassen 2014), and enhance sediment accretion (Koch et al. 2013). Seagrasses have also recently been identified as a major carbon sink, responsible for 10-18% of the Ocean's carbon accumulation (McLeod et al. 2011; Fourqurean et al. 2012; Greiner et al. 2013).

Many seagrasses are intertidal species which grow in shallow coastal lagoons and are therefore exposed to large variations in light and sediment loading/resuspension (Harlin 1995). Additionally, seagrasses are exposed to large fluctuations in oxygen levels, ranging from 71 to 311 μmol L<sup>-1</sup> under normal conditions (Brodersen et al. 2017) and as low as 10% (approx. 20 μmol L<sup>-1</sup>) air saturation during night time in areas where seagrass die-offs were observed (Borum et al. 2005). They also have anatomical adaptations such as the absence of stomata and the development of extensive aerenchyma (Penhale and Wetzel 1983) along with physiological

adaptations such as the ability to tolerate hypoxic and anoxic conditions especially in the roots and rhizomes (Pregnall et al. 1984; Papenbrock 2012) which they possibly inherited from submerged freshwater ancestors (Les et al. 1997). As seagrass persistence generally require a large flux of photosynthetically active radiation, roughly 10% of surface irradiance (Papenbrock 2012), the effects of light on seagrass ecology have been extensively studied (Ralph et al. 2007; Staehr and Borum 2011; Brodersen et al. 2015; Chartrand et al. 2016). However, less attention has been given to the effects of photosynthetic gases and associated metabolic pathways in seagrasses, e.g., the effects of low O<sub>2</sub> conditions have only been reported in a few papers to our knowledge (e.g. Black et al. 1976; Downton et al. 1976; Beer et al. 2002; Greve et al. 2003; Buapet et al. 2013).

The photosynthetic processes of seagrasses are very similar to that of other angiosperms (Beer et al. 1998). Most seagrasses were classified biochemically as C<sub>3</sub> plants on the biochemical criteria of short-term inorganic <sup>14</sup>C incorporation products and the ratio of Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) to phosphoenolpyruvate carboxylase (PEPC) activities, although *Thalassia testudinum* has C<sub>4</sub> metabolism (Benedict and Scott 1976) and *Halophila stipulacea* has C<sub>3</sub>-C<sub>4</sub> intermediate metabolism by these criteria (Beer et al. 1980; Beer et al. 2002; Koch et al. 2013). The C<sub>3</sub> CO<sub>2</sub> fixation process begins with RuBisCO which catalyses the carboxylation using CO<sub>2</sub> (and H<sub>2</sub>O) of ribulose 1, 5-bisphosphate (RuBP), producing two of the 3 carbon molecules; 3-phosphoglycerate (PGA) as the initial stable product. In parallel there is the oxygenation of RuBP with O<sub>2</sub> to yield one PGA and one 2-phosphoglycolate. The ratio of carboxylase to oxygenase activity is determined by the kinetic properties of the molecular form of RuBisCO involved and the CO<sub>2</sub>:O<sub>2</sub> ratio at the active site of RuBisCO.

The C<sub>4</sub> carbon fixation process on the other hand, begins with the carboxylation (using HCO<sub>3</sub><sup>-</sup> as the immediate inorganic C substrate) of phosphoenolpyruvate (PEP) in the RuBisCO-free cytosol, forming the 4-carbon acid oxaloacetate as the initial stable product. Oxaloacetate is subsequently converted to malate and/or aspartate which are moved to, and decarboxylated in, the compartment containing RuBisCO, generating CO<sub>2</sub> which is used in the carboxylation of RuBisCO, and a 3-carbon acid that returns to the cytosol, regenerating PEP. C<sub>4</sub> photosynthesis involves a higher steady-state CO<sub>2</sub> concentration than that available to RuBisCO in biochemically and physiologically defined C<sub>3</sub> photosynthesis with diffusive entry of CO<sub>2</sub> from the bulk external medium to RuBisCO, i.e., C<sub>4</sub> photosynthesis acts as a CO<sub>2</sub> concentrating mechanism (CCM). The regeneration of PEP to allow further PEPC activity is an energy (as ATP) requiring process. The C<sub>4</sub> mechanism has an advantage over the C<sub>3</sub> mechanism in low CO<sub>2</sub> and/or high O<sub>2</sub> environments as PEPC reacts specifically with CO<sub>2</sub> (after its conversion to HCO<sub>3</sub><sup>-</sup>) with no interference from O<sub>2</sub> and with accumulation of CO<sub>2</sub> around RuBisCO that largely suppresses RuBisCO oxygenase activity. In C<sub>3</sub> plants, to a much smaller extent, the 2-phosphoglycolate from the oxygenase reaction is metabolised to PGA and then sugar phosphates in energy-requiring photorespiratory carbon oxidation cycle (PCOC); whether the oxygenase-PCOC combination physiologically decreases the energetic efficiency of C<sub>3</sub> plants relative to C<sub>4</sub> plants depends on the energy cost of oxygenase-PCOC relative to the cost of operating the C<sub>4</sub> pathway with its inevitable leakage of CO<sub>2</sub> from the pool accumulated around RuBisCO (Raven 2014; Raven and Beardall 2016; Larkum et al. 2017). It is important to note that the occurrence of any CCM gives physiological (not biochemical) characteristics similar to those of a C<sub>4</sub> plant (Raven 2014; Raven and Beardall 2016, and references therein; Larkum et al. 2017). Thus, photosynthesis that is relatively insensitive to O<sub>2</sub>, has a high affinity for CO<sub>2</sub> and can deplete the CO<sub>2</sub> in a closed system to very low

concentrations and is not diagnostic of C<sub>4</sub> photosynthesis since it could also result from any CCM activity.

In marine plants, inorganic carbon species diffusion to the leaf surface has a greater potential to restrict the rate of photosynthesis due to the slower diffusion rates in water. Further, the primary form of dissolved inorganic carbon in seawater is HCO<sub>3</sub><sup>-</sup> (90%) with CO<sub>2</sub> making up about 1% (see below). Hence a CCM would be advantageous for marine plants, for example, physiologically “C<sub>4</sub>-like” inorganic carbon acquisition has been observed in the seagrass *Zostera noltii* based on high light saturation values and a lack of observable photorespiration (Raven 1984; Jiménez et al. 1987); however, as noted by Raven (1984), this is not diagnostic of C<sub>4</sub> rather than some other CCMs. By contrast Waghmode and Joshi (1983), using *Halophila beccarii* (as *H. beccaeii*), showed short-term inorganic <sup>14</sup>C labelling of aspartate, and also alanine, i.e. features of C<sub>4</sub> photosynthesis, although critics could say that the labelling period was not short enough to show the real initial product of inorganic C assimilation. Despite this, short-term inorganic <sup>14</sup>C labelling is the most conclusive methods to indicate C<sub>4</sub> photosynthesis in seagrasses. The high ratio of PGA phosphatase to 2-phosphoglycolate phosphatase activity in *H. beccarii* (Waghmode and Joshi 1983) is consistent with a decreased RuBisCO oxygenase generating 2-phosphoglycolate, and hence decreased flux through the PCOC producing glycine and serine, and a requirement for the non-phosphorylated pathway from photosynthetic or glycolytic PGA to glycerate and hence to serine and glycine. However, the decreased RuBisCO oxygenase and concomitant requirement for the PGA to glycerate pathway to serine and glycine could occur in any organism with a CCM. Colman and Norman (1997) showed the occurrence of a phosphorylated pathway, not involving PGA phosphatase, from PGA to serine in cyanobacteria with CCMs and minimal 2-phosphoglycolate synthesis. The occurrence of PEPC and aspartate aminotransferase in *H. beccarii* does not signify a C<sub>4</sub> pathway, since these enzymes are ubiquitous in plants (Aubry et al. 2011). HCO<sub>3</sub><sup>-</sup> is the predominant inorganic C

species in seawater (at pH 8.16: 85.8%  $\text{HCO}_3^-$ , 0.4%  $\text{CO}_2$  and 13.7%  $\text{CO}_3^{2-}$ , Pierrot et al. 2006) with  $\text{CO}_2$  at about the same concentration ( $\text{mol m}^{-3}$  of fluid medium) as in air. However, the diffusion coefficient for  $\text{CO}_2$  in water is about  $10^{-4}$  that in air (Raven 1984) so, despite the usually thinner diffusion boundary layer in water (~0.01-0.1 mm) than in air (~1 mm) under ecologically relevant conditions (Vogel 1994),  $\text{CO}_2$  diffusion to the leaf surface, and  $\text{O}_2$  diffusion from the leaf surface (Mass et al. 2010) may limit photosynthesis in marine plants more than in land plants. Although the diffusion coefficient for  $\text{HCO}_3^-$  is lower than that of  $\text{CO}_2$  (Raven 1984), the quantitative predominance of  $\text{HCO}_3^-$  in seawater means that it can support a larger flux to the leaf surface in response to a given potential for  $\text{CO}_2$  assimilation in the leaf, provided the leaf can use  $\text{HCO}_3^-$ . Some seagrasses can utilise  $\text{HCO}_3^-$  either directly via active transport into epidermal cells (Beer and Rehnberg 1997), or more commonly, indirectly by dehydrating  $\text{HCO}_3^-$  to  $\text{CO}_2$  via the enzyme carbonic anhydrase (CA) in the epidermal cell wall (Beer et al. 1980) usually interacting with co-localised leaf surface acidification by energy-requiring  $\text{H}^+$  efflux (Hellblom et al. 2001; Hellblom and Axelsson 2003; Borum et al. 2016). Such indirect methods for enhancing inorganic C ( $\text{C}_i$ ) uptake make predictions of  $\text{C}_4$  mechanisms in seagrasses doubtful unless supported by strong evidence.

The suggestion that  $\text{C}_4$  photosynthesis does not occur in seagrasses because of the absence of bundle sheath cells containing chloroplasts and the lack of true Kranz anatomy (Beer et al. 1980) has subsequently been shown to be invalid. Some freshwater submerged aquatic plants and some terrestrial  $\text{C}_4$  members of the Chenopodiaceae utilise  $\text{C}_4$  photosynthesis via the fixation of external inorganic C by PEPC, and the fixation of  $\text{CO}_2$  (regenerated from  $\text{C}_4$  acid decarboxylation) by RuBisCO, the carboxylases occurring in the cytosol and the chloroplasts respectively of a single cell (Voznesenskaya et al. 2001; references in Raven and Beardall 2016).



With recent advances in genomics and transcriptomics, researchers have the capacity to examine molecular mechanisms which drive seagrass photosynthesis to an extent that was unimaginable just a decade ago. In this context, the presence of genes encoding enzymes characteristic of the C<sub>4</sub> carbon fixation pathway in seagrass transcriptome could provide evidence relevant to the argument that seagrasses are not strictly C<sub>3</sub> plants.

For instance, several genes coding for PEPC, a cytosolic enzyme essential for the C<sub>4</sub> carbon fixation pathway in higher plants (Chollet et al. 1996), have been identified in the transcriptome of *Zostera muelleri* (unpublished data). This enzyme catalyses the irreversible  $\beta$ -carboxylation of phosphoenolpyruvate (PEP) by HCO<sub>3</sub><sup>-</sup> to produce oxaloacetate (as described previously), a key intermediate in the C<sub>4</sub> carbon fixation pathway. However, PEPC has a ubiquitous anaplerotic role in plants and algae (excluding dinoflagellates where PEPC is replaced by pyruvate carboxylase) in replenishing the intermediates of the Krebs cycle depleted by the use of oxaloacetate and 2-oxoglutarate in the synthesis of some amino acids and of pyrimidines haems and chlorins (Raven 1984; Raven and Farquhar 1990; Aubry et al. 2011; Raven 2014). Additional PEPC expression is needed in the roots of seagrasses growing on carbonate substrata in the production of organic acids that release phosphate from apatite in the carbonate sediment (Long et al. 2008; Raven 2014). Chi et al. (2014) show that there is at least 1 copy of each of 8 genes related to C<sub>4</sub> photosynthesis and also to other aspects of metabolism in the 4 completely sequenced tracheophytes (2 with C<sub>3</sub> photosynthesis, 2 with C<sub>4</sub> photosynthesis) and 1 completely sequenced C photosynthesis bryophyte. For PEPC, the 2 C<sub>4</sub> plants had 3 or 4 copies of the gene, while the 2 C<sub>3</sub> plants have 4 or 6 copies. Therefore, the presence of the PEPC gene in the transcriptome of *Zostera muelleri* (unpublished data) does not show that *Z. muelleri* is other than, biochemically, a C<sub>3</sub> plant.

Furthermore, genes encoding CA were also detected in the *Z. muelleri* transcriptome

(unpublished data). CA catalyses the reversible interconversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  ( $\text{HCO}_3^- + \text{H}^+ = \text{CO}_2 + \text{H}_2\text{O}$ ). CAs are also involved in several non-photosynthetic reactions in plants (Raven 2014 and references therein), possibly including provision of respiratory  $\text{CO}_2$  to  $\text{HCO}_3^-$  for the PEPC activity (Raven 2014) required for synthesis of the organic acids used, after secretion, in phosphate release from carbonate substrata (Long et al. 2008; Raven 2014) in seagrass roots. One or more CAs are components of  $\text{C}_4$ -based and other CCMs, as well as in  $\text{C}_3$  photosynthesis (Aubry et al. 2011; Raven 2014; Raven and Beardall 2016).

However, expression of  $\text{C}_4$  photosynthetic metabolism in some submerged freshwater relatives of seagrasses is a function of environmental conditions (low  $\text{CO}_2$ , high  $\text{O}_2$ ), unlike terrestrial  $\text{C}_4$  plants where it is constitutive (references in Raven and Beardall 2016).

Therefore it is possible that the expression of some PEPC and CA genes in seagrasses varies with the  $\text{O}_2$  concentration and hence the potential for RuBisCO oxygenase activity, noting that the other light dependent  $\text{O}_2$  consuming reactions, i.e. the water-water (or oxygenoxygen) cycles of the Mehler Peroxidase reaction and of the oxidation by the plastid terminal oxidase of plastoquinone reduced by PSII, are minimal in the only seagrass (*Zostera marina*) investigated: Buapet and Björk (2016).

The aim of this molecular-physiological study was to address the following: (i) how the photosynthetic and respiratory rates are affected by experimentally reduced  $\text{O}_2$  concentration in the water column using electrochemical microsensors and (ii) how this reduced  $\text{O}_2$  concentration affects the expression levels of PEPC and CA using Reverse Transcription quantitative real-time PCR (RT-qPCR).

## **MATERIAL AND METHODS**

### Seagrass collection and experimental setup

Specimens of *Zostera muelleri* ssp. *capricorni* (Asch) S. W. L. Jacobs and attached marine sediment were collected from Pittwater, NSW, Australia (33° 38' 45.6"S, 151° 17' 12.8"E) on the 14<sup>th</sup> of May 2015. In order to mimic the conditions of Pittwater at the University of Technology Sydney (UTS) aquarium facility, salinity and temperature of the water were measured in the field; ambient salinity: 31 and water temperature: 22°C, along with rapid light curves of *Z. muelleri* to determine suitable light conditions. Rapid light curves measured in the field on 3 *Z. muelleri* plants using a Diving-Pulse Amplitude Modulated (PAM) fluorimeter (DIVING-PAM, Heinz Walz GmbH, Eichenring, Germany) indicated that photosynthetic saturating light was approximately 230  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , which is consistent with saturating light levels previously found for this seagrass species in temperate regions (Schwarz 2004; Bulmer et al. 2016). Collection was performed at low tide in shallow water (~1 m) and plants were transported immediately to an aquarium facility at the University of Technology Sydney. Before further handling, the specimens and sediment were placed into aquaria for 48 hours, after which they were separated into individual ramets/shoots (see Procaccini et al. 2007). These samples were then acclimated for 2 months in 40 L glass aquaria to conditions mirroring that of the sampling site e.g.: a salinity of 31, temperature of 22±1°C and illumination with an incident photon irradiance of ~230  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (12 h : 12 h light : dark cycle). Sixteen individual shoots of *Z. muelleri* were then transplanted in pairs into 8 plastic tanks (~150 mm in diameter, 4 tank replicates per treatment) with ~30 mm of sediment and acclimated at the same environmental conditions as above for 2 weeks.

The experiment was initiated in the middle of the photoperiod. Each of the treatments (i.e. control and low O<sub>2</sub>) had one 100 L sump (100 L plastic bin) underneath a 40 L aquaria/table that held experimental plastic containers (Supplementary Figure S1). From each sump, water with corresponding O<sub>2</sub> level was pumped into four replicate plastic containers resulting in a total of 8 containers (4 replicate containers x 2 sumps/O<sub>2</sub> treatments = 8 containers in total). Each container contained 2 individual shoots of *Z. muelleri* (8 tanks x 2 shoots = 16 shoots). For the low O<sub>2</sub> treatment, the dissolved O<sub>2</sub> was lowered in the sump over an interval of 1 h via flushing with nitrogen gas (CO<sub>2</sub>-free) to an average O<sub>2</sub> concentration of ~9 μmol O<sub>2</sub> L<sup>-1</sup> as measured by a calibrated dissolved O<sub>2</sub> probe (FDO 925, WTW GmbH, Germany). The dissolved O<sub>2</sub> concentration within the aquaria was maintained between 9 and 16 μmol O<sub>2</sub> L<sup>-1</sup> for the duration of the experimental period of 24 h (Supplementary Figure S2). Deviations from the initial pH of 8.16 (+/-0.01 pH) was controlled via bubbling of 99.9% pure CO<sub>2</sub>, which was automatically controlled by a calibrated pH/CO<sub>2</sub> controller (7074/2, TUNZE Aquarientechnik GmbH, Germany, Supplementary Figure S3). The control tank set up was the same except for bubbling air instead of N<sub>2</sub> and CO<sub>2</sub>.

#### Oxygen measurement setup

The lower half of leaf 2 (~20 mm) were cut from 3 individual ramets/shoots (see Procaccini et al. 2007) randomly picked from a pool of untreated samples which were previously subjected to the same acclimation procedure. The leaf sections were cleaned of any epiphytes and then fixed in place with fine pins on a piece of styrofoam in a custom-made flowchamber (see Brodersen et al. 2014). The sections were angled in such a way as to allow for unobstructed flow over each of the sampling areas of the leaves. Illumination of the leaves to the desired light levels was achieved via a fibre-optic tungsten halogen lamp (KL-2500LCD, Schott GmbH, Germany) with the irradiance measured at the leaf surface using a 4π quantum sensor (US-SQS/L, Walz GmbH, Germany) connected to a calibrated light meter (LI-250A,

LI-COR Inc., USA). Seawater was pumped through the flow chamber at a constant rate of  $\sim 5 \text{ mm s}^{-1}$  for the duration of the experiment. Atmospheric air was bubbled during the control phase of the experiment, while nitrogen gas was bubbled during the treatment phase of the experiment, lowering the  $\text{O}_2$  concentration from  $\sim 231 \mu\text{mol O}_2 \text{ L}^{-1}$  to  $\sim 9 \mu\text{mol O}_2 \text{ L}^{-1}$  (as described above). Salinity, temperature and pH of the seawater were kept constant throughout the experiment.

Vertical  $\text{O}_2$  concentration micro-profiles towards the leaf tissue surface (approx.  $0.031 \text{ cm}^2$ ) and thus across the diffusive boundary layer (DBL) were recorded using a Clark-type  $\text{O}_2$  microsensor (OX-50, tip diameter approx.  $50 \mu\text{m}$ ; Unisense A/S Aarhus, Denmark; Revsbech, 1989) with a fast response time ( $< 0.5 \text{ s}$ ) mounted on a motorized micromanipulator (Unisense A/S, Aarhus, Denmark). The microsensor was connected to a multimeter (Unisense Microsensor Multimeter A/S. Aarhus, Denmark) and interfaced with a PC running dedicated data acquisition and positioning software (SensorTrace PRO; Unisense A/S, Aarhus, Denmark). The microsensor was positioned at the leaf tissue surface (defined as  $0 \mu\text{m}$ ) manually by observing the microsensor tip and leaf tissue surface through a stereomicroscope mounted on an articulating arm. Subsequent measurements of vertical  $\text{O}_2$  concentrations for micro-profiles were measured at  $100 \mu\text{m}$  increments using the motorised micromanipulator (Unisense Motorised Micromanipulator A/S, Aarhus, Denmark) controlled by dedicated positioning software (SensorTrace PRO). Linear calibration of the  $\text{O}_2$  microsensor was obtained from signal readings in 100% air-saturated seawater and anoxic seawater (seawater amended via  $\text{N}_2$  bubbling and the  $\text{O}_2$  scavenger sodium dithionite) at experimental salinity, pH and temperature.

Seagrass maximum quantum efficiency of photosystem II ( $F_v/F_m$ ; Baker 2008) values were measured regularly on 3 biological replicates using a Pulse Amplitude Modulated (PAM) fluorimeter (Pocket PAM, Gademann Instruments, Wuerzburg, Germany, see Figueroa et al.

2013) after dark-adaptation for ~10 min. Minimal fluorescence ( $F_0$ ) was recorded using a weak measuring light, which was then followed by a saturating pulse (irradiance of 3,500  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  for 0.8 s) to measure maximal fluorescence ( $F_m$ ). Under these conditions,  $F_v/F_m$  ratios provides a measure of maximal PSII photochemical efficiency ( $F_v = F_m - F_0$ ) and were, in this experiment, used as an indicator of seagrass maximum quantum efficiency of PSII during experimentation.

#### Photosynthesis-Irradiance (P-I) curves

Established methods for determining rates of photosynthesis in marine plants via  $\text{O}_2$  microsensors were used in this study (see Jørgensen and Revsbech 1985; Kühl et al. 1995; Lichtenberg and Kühl 2015; Pedersen et al. 2016). The effective DBL thickness was estimated by extrapolating the linear  $\text{O}_2$  concentration gradient until it intersects with the constant  $\text{O}_2$  concentration in the overlaying water. These  $\text{O}_2$  micro-profiles determined at the leaf tissue surface in the flow chamber were measured at incident photon irradiances of 0, 25, 50, 100, 200, 500 and 700  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , first under control conditions (aerated seawater, 31 salinity,  $22 \pm 1^\circ\text{C}$  and pH 8.16). Leaves were then exposed to treatment conditions ( $\sim 9 \mu\text{mol O}_2 \text{ L}^{-1}$ , 31 salinity,  $22 \pm 1^\circ\text{C}$  and pH 8.16) at the beginning of the subsequent light cycle for 3 h before measurements were taken at incident photon irradiances in the following order: 25, 50, 100, 200, 500, 700 and 0  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Leaves were allowed to equilibrate to each of the incident photon irradiances for 30 mins before  $\text{O}_2$  microprofiles were recorded.

Based on the measured  $\text{O}_2$  concentrations around the leaf tissue surface,  $\text{O}_2$  fluxes were calculated using Fick's first law of diffusion:

$$(1) J_{\text{O}_2} = -D_0 \frac{\Delta C}{\Delta Z}$$

where  $D_0$  is the diffusion coefficient of  $\text{O}_2$  in seawater at the experimental salinity and temperature ( $2.2088 \times 10^{-5} \text{ cm}^{-2} \text{ s}^{-1}$ ; tabulated values taken from { HYPERLINK

"http://www.unisense.com/" \h }{ HYPERLINK "http://www.unisense.com/" \h } and  $\frac{\Delta C}{\Delta z}$  is the gradient of the linear O<sub>2</sub> concentration within the DBL.

The measured O<sub>2</sub> fluxes across the leaf surface, which are the equivalent of rates of net photosynthesis, were then fitted with an exponential saturation model (Webb et al. 1974; Lichtenberg and Kühl 2015) using OriginPro (OriginLab, USA) with the added respiration term,  $R$ , to account for O<sub>2</sub> consumption (Spilling et al. 2010):

$$(2) P(E) = P_{max} \left( 1 - \exp^{\frac{-\alpha}{P_{max}}} \right) + R$$

where  $\alpha$  is the initial slope of the P-I curve in the light-limiting phase,  $P_{max}$  is the maximum net photosynthetic rate and  $R$  is the respiration term.

This allowed for calculations of the minimum photosynthetic saturation irradiance ( $E_k$ ), which gives an indication of the onset of photosynthesis saturation, and the compensation irradiance ( $E_c$ ), that is, where the O<sub>2</sub> produced via photosynthesis equals the respiratory demands, using the following equations (e.g. Lichtenberg and Kühl 2015):

$$(3) E_k = \frac{P_{max}}{\alpha}$$

$$(4) E_c = \frac{P_{max} \log_{10} \left( \frac{R}{P_{max}} + 1 \right)}{-\alpha}$$

#### Sample collection, RNA extraction and cDNA synthesis

Four biological replicate samples of *Z. muelleri* were randomly collected for each time point (0 and 24 h) and for each treatment (control and low O<sub>2</sub>). Samples included above-ground tissue (i.e. leaf biomass) only, as this part of the plant is likely to respond more immediately, being photosynthetically active and in direct contact with molecular O<sub>2</sub> in the water-column.

Samples were packed in aluminium foil envelopes and snap-frozen directly in liquid nitrogen. Samples were stored at -80°C for 15 days prior to further RNA extraction and RT-qPCR analysis. Briefly, for each sample, ~70 mg of freeze-dried leaf biomass was grounded into powder using a mortar and pestle in liquid nitrogen. RNA was then extracted using the PureLink RNA Mini Kit (Ambion) following manufacturer's instructions. Column purification DNase digestion was carried out using PureLink DNase Set (Ambion) following the manufacturer's instructions. The RNA quantity and quality was assessed using a spectrophotometer (NanoDrop 2000) and absorbance at 260/280 nm. Good quality RNA samples were stored at -80°C for further RT-qPCR experiments. A total of 500 ng of good quality RNA was used for each sample for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's instructions. The resulting cDNA samples were diluted 1:20 for use in RT-qPCR analysis.

#### Primer design

The present study conforms to the Minimum Information for Publication of Quantitative Real-Time PCR guidelines (Bustin et al. 2009). In this section, we indicate the essential information, *sensu* Bustin et al. (2009), required to allow reliable interpretation of the corresponding RT-qPCR results.

In-depth analysis of *Zostera muelleri* Transcriptomics Database (Hayward et al, in prep), revealed transcripts encoding proteins with high similarities to the domains of PEPC and CA proteins already identified in the seagrass *Zostera marina* (Olsen et al. 2016). It is interesting to note that the genome of *Z. muelleri*, which was not available at the time of this study, has been published since (Lee et al. 2016). The functional domains of three of these sequences, coding for PEPC1 (KMZ56135), PEPC2 (KMZ58048) and  $\square$ -CA (KMZ56166) respectively, were used as a template to design sequence-specific primers for RT-qPCR using the software, Primer3 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) with default settings. The



sizes of the resulting amplicons were kept from 79 to 195 bp (Table 1) ensuring similar PCR efficiencies and facilitating cross comparison of assays. The specificity of each selected primer pair was observed by PCR amplification as single bands at the expected size resolved via agarose gel electrophoresis.

#### Reverse Transcription Quantitative Real Time - PCR and Gene Expression Analysis

SYBR green PCR master mix (Warrington, Cheshire, UK) was used for RT-qPCR assays in 96-wells plates in a Step One Plus<sup>TM</sup> Real-Time PCR System (Applied Biosystems, USA). PCR conditions were: initial denaturation of 10 min at 95°C, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s and 68°C for 30 s. A dissociation step was included at the end: 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The final reaction volume was 10 µL, including 0.8 µL of primers (Table 1) and all reactions were conducted in technical triplicates. The RTqPCR efficiency for each gene and each treatment was determined from a cDNA dilution gradient of 27, 9, 3 and 1 ng and a linear regression model (Pfaffl 2001). The corresponding RT-qPCR efficiencies were calculated according to the equation below (Radonić et al. 2004):

$$(5) \text{ PCR efficiency} = (10^{\left[-\frac{1}{\text{slope}}\right]} - 1) \times 100$$

All the RT-qPCR efficiencies obtained with the different primers were between 96-104%, with a calibration coefficients >0.969 (Table 1, see Supplementary Figure S4). A no template control, as well as a no reverse transcription control was generated for each gene and each treatment to ensure that the PCR reactions were free of DNA contamination.

Data from RT-qPCR was analysed using the Step One Plus<sup>TM</sup> Software (Ver. 2.3; Applied Biosystems). Expression levels were determined as the number of cycles needed for the amplification to reach a fixed threshold in the exponential phase of the RT-qPCR reaction. The

cycle threshold ( $C_T$ ) was set at 0.03 for all genes. To quantify changes in target genes expression,  $C_T$  were imported then transformed into quantities using corresponding RT-qPCR efficiency to obtain Normalized Relative Quantities.

#### Selection of reference genes

In order to select the best reference genes for the experimental conditions, expression stability was analysed using NormFinder (Andersen et al. 2004). The corresponding  $C_T$  values were used directly in the software package NormFinder (Andersen et al. 2004) to rank and select the most stable reference genes. Candidate reference genes and corresponding primers used in this study were identified previously (Schliep et al. 2015). Because these candidates reference genes were initially validated under low light stress conditions, we used NormFinder to measure their stability value during low  $O_2$  exposure (i.e. direct measure for the estimated expression variation) as previously described by (Andersen et al. 2004). We also ran complementary analysis using a second software (GeNorm, Vandesompele et al. 2002) which led to similar results as for NormFinder. According to Normfinder, the most stable genes under our experimental conditions were GADPH, Actin and S4 and the best combination of two reference genes: S4 and GADPH (see Supplementary Figure S5) was then used to normalize target gene expression profile in *Z. muelleri* under low  $O_2$ .

#### Statistical analyses

Statistical analyses were performed using a Repeated Measures Analyses of Variance with PERMANOVA+ software in PRIMER v6 (Anderson et al. 2008). The analyses tested the null hypothesis that there is no difference in the  $\alpha$ ,  $P_{max}$ ,  $R$ ,  $E_k$  and  $E_c$  values derived from the fitted P-I curves of the control and low  $O_2$  treated leaves. The RT qPCR data was analysed in the same way to test the null hypothesis that there is no difference in the normalized relative quantities of PEPC1, PEPC2 and  $\gamma$  CA in control and low  $O_2$  treated plants. We randomized

our sampling within each treatment to minimize lack of independence and to separate the two O<sub>2</sub> levels from other potential effects originating from containers location on the table. Throughout this paper, values given for microsensor data are the mean of 3 biological replicates, while RT-qPCR data are the mean of 4 biological replicates, including technical triplicates. Results were considered significant at 5%.

## RESULTS

### Rates of net photosynthesis and P-I curves

The vertical O<sub>2</sub> concentration micro-profiles showed a ~0.02 cm thick DBL at the leaf surface of *Z. muelleri* at all irradiances tested in both the control and low O<sub>2</sub> conditions (Figure 1). The average O<sub>2</sub> concentration at the leaf tissue surface of the control plants increased from 203 to 352 μmol O<sub>2</sub> L<sup>-1</sup> as a response to an increasing incident irradiance from 0 to 100 μmol photons m<sup>-2</sup> s<sup>-1</sup>, as compared to an increase from 3.6 to 187 μmol O<sub>2</sub> L<sup>-1</sup> in the low O<sub>2</sub>-treated plants. This translated to statistically different O<sub>2</sub> flux values between the control and low O<sub>2</sub> treated plants at incident photon irradiances of 0, 25 and 50 μmol photons m<sup>-2</sup> s<sup>-1</sup> (Permutational t-test,  $t_4 = 4.7575, P = 0.0026$ ;  $t_4 = 12.526, P = 0.0002$ ;  $t_4 = 5.4299, P = 0.0032$  respectively, Figure 2.). Comparison between the dark respiration rates (R) and the initial slope of the P-I curve ( $\alpha$ ), which gives an indication of photosynthetic activity, of the control and low O<sub>2</sub> treated plants also indicated statistical difference (Permutation t-test between control and low O<sub>2</sub> treated plants for R and  $\alpha$ ;  $t_4 = 6.8879, P = 0.0018$ ;  $t_4 = 5.2964, P = 0.0064$  respectively, Table 2); with a decrease in R but an increase in  $\alpha$ .

In contrast, between incident irradiances of 200 to 700 μmol photons m<sup>-2</sup> s<sup>-1</sup>, there was only a slight increase of ~2 μmol O<sub>2</sub> L<sup>-1</sup> in the control plants, from 386 to 388 μmol O<sub>2</sub> L<sup>-1</sup>, and an increase of ~50 μmol O<sub>2</sub> L<sup>-1</sup> in the low O<sub>2</sub> treated plants from 194 to 246 μmol O<sub>2</sub> L<sup>-1</sup>. This translated to net oxygen flux values that were not statistically different among the 200, 500 and 700 μmol photons m<sup>-2</sup> s<sup>-1</sup> irradiance treatments (Permutational t-test,  $t_4 = 2.2527, P = 0.088$ ;  $t_4 = 0.65804, P = 0.5314$ ;  $t_4 = 1.147, P = 0.306$  respectively, Figure 2). The calculated P<sub>max</sub> values in the control and low O<sub>2</sub> treated plants, derived from the fitted P-I curves, were also not statistically different (Permutational t-test,  $t_4 = 0.2956, P = 0.7974$ , Table 2).

Calculations of the  $E_k$  of *Z. muelleri* plants, in the control and low  $O_2$  conditions, yielded an average saturation irradiance of ~60 and 51  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  respectively (Table 2), values that are not statistically different (Permutational t-test,  $t_4= 1.2571$ ,  $P= 0.2892$ , Table 2); however, there was a statistically significant decrease in the  $E_c$  between the control plants and low  $O_2$  treated plants (Student's t-test,  $t_4= 6.5624$ ,  $P= 0.0012$ , Table 2) which were calculated to be at incident irradiances of ~5.19 and 0.71  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , respectively (Table 2).

#### Expression levels of target genes

Among the 7 candidate reference genes tested, the best combination of two reference genes included S4 and GADPH gene ( $M=0.185$ ; see Supplementary Figure S5). These two reference genes were then used to evaluate target gene expression profile in *Z. muelleri* under low  $O_2$ . The relative quantification demonstrated a significant down-regulation of the PEPC-1 and  $\gamma$ -CA genes for seagrass incubated in low  $O_2$  when compared to control (Figure 3). Specifically, there was a ~2.2 fold decrease in PEPC-1 gene expression in low  $O_2$  treated samples relative to control (Permutational t-test,  $t_6= 2.9916$ ,  $P= 0.0188$ , Figure 3). Similarly, a ~2.8 fold decrease was observed in  $\gamma$ -CA (Permutational t-test,  $t_6= 3.3414$ ,  $P= 0.0072$ ; Figure 3). No statistical difference was observed in the expression level of the PEPC-2 gene (Permutational t-test,  $t_6$ ,  $P= 0.0664$ , Figure 3), although a decreasing trend was observed in low  $O_2$  treated samples when compared to the controls.

## DISCUSSION

### Photosynthetic parameters

We observed that by lowering the  $O_2$  concentration in the water-column, the net photosynthetic rates of *Zostera muelleri* were enhanced in the light limited region of the P-I curve (Figure 2). Further, we confirmed that these results were not due to impacts on the health of the *Z. muelleri*

photosystems as shown by the  $F_v/F_m$  which remained  $\geq 0.7$  for the duration of the experimental period (as measured via PAM-fluorometry, see Supplementary Figure S6). This shows that *Z. muelleri* has a higher photosynthetic activity with an increased  $CO_2:O_2$  ratio; conditions which potentially could lead to a decreased oxygenase activity of RuBisCO and decreased flux through the PCOC, while increasing carboxylation. Similar studies involving other marine angiosperms such as *Cymodocea rotundata*, *Zostera marina* and *Ruppia maritima* support our findings since the net photosynthetic rate in these plants also increased in response to reduced ambient  $O_2$  concentrations (Black et al. 1976; Downton et al. 1976; Beer et al. 2002; Buapet et al. 2013) whereas low  $O_2$  conditions had no effect on the gross photosynthetic rate of a green alga *Ulva intestinalis* due to suppressed photorespiration (Drechsler and Beer 1991; Beer et al. 2000) via the maintenance of a carbon concentrating mechanism (Björk et al. 1993; Larsson et al. 1997). In addition, within the lacunae of seagrass leaves in light conditions, Carlson et al. (1988) and Roberts and Moriarty (1987) found that  $O_2$  accounted for 38% of the gas within these lacunae, leading to the hypothesis that seagrass leaves may possess a mechanism to minimise photorespiration by inhibiting the accumulation of intracellular  $O_2$ . These data show the photosynthetic behaviour of *Z. muelleri* is typical of a plant with  $C_3$  biochemistry and physiology in low  $O_2$  conditions.

We also found a statistically significant decrease in the dark respiration and the subsequent compensation irradiance of the low  $O_2$  treated leaves (Table 2), which was not so surprising owing to the low ambient  $O_2$  availability. A similar effect has been observed in the dark respiration of *Z. marina* when exposed to low  $O_2$  conditions (Buapet et al. 2013). This is believed to be the result of the reduced  $O_2$  availability in the surrounding environment and since  $O_2$  transport to the lacunae ceases after 15 to 30 mins following the onset of darkness (Smith et al. 1984) and the  $O_2$  concentration of the air in the lacunae drops down to  $<1\%$  of the air around the leaf (Carlson et al. 1988), respiration is decreased. Hence, respiration is an important

factor to take into account when interpreting photosynthetic activity and efficiency responses to changing environmental conditions. Indeed, the effect of low O<sub>2</sub> levels on net photosynthetic rates seen in this study could be solely due to a decreased rate of respiration, particularly as the only statistically significant differences between control and low O<sub>2</sub> treatments were in the 0, 25 and 50 μmol photons m<sup>-2</sup> s<sup>-1</sup> irradiances where respiration strongly affects the rate of net photosynthesis, while no statistical difference is seen at the higher irradiances where photorespiration would be expected to have stronger effects (Beardall et al. 2003).

While there appears to be a slight increasing trend in the rate of photosynthesis in the light saturated section of the P-I curve, there was no statistical difference in the saturating irradiance and maximum net photosynthetic rate between the low O<sub>2</sub> treated and control leaf fragments (Figure 2; Table 2). As the pH (and subsequently dissolved inorganic carbon; DIC) was maintained at 8.16 throughout the experiment, our results suggest that the growth of *Z. muelleri* was C-limited and this is consistent with previous findings on other seagrass species (Björk et al. 1997; Zimmerman et al. 1997). With regards to the DIC in seawater, speciation depends on the salinity and temperature, but the main form present at pH 8.16 is HCO<sub>3</sub><sup>-</sup> (Pierrot et al. 2006), and HCO<sub>3</sub><sup>-</sup> is expected to be the major inorganic C source for photosynthesis in seagrasses. Additional experimentation have shown seagrass to be capable

of utilizing HCO<sub>3</sub><sup>-</sup> by means other than uncatalysed conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> in the DBL

(Larkum et al. 2017), such as *Halophila stipulacea*, *Thalassodendron ciliatum*, *Halodule uninervis* and *Syringodium isoetifolium* (Beer et al. 1977; Koch et al. 2013; Borum et al.

2016), however the exact method of HCO<sub>3</sub><sup>-</sup> uptake remains unclear (Larkum et al. 2017).

Moreover, at high photon irradiances O<sub>2</sub> produced via photosynthesis results in similar O<sub>2</sub> microclimates in and around leaves within both treatments, owing to an internal and external build-up of O<sub>2</sub> as a result of the leaf DBLs impeding gas exchange with the surrounding water column (Brodersen et al. 2015). This may therefore explain the similar maximum net

photosynthesis rates measured in the low O<sub>2</sub> and control treatment at photon irradiances  $\geq 100$   $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (Fig. 1-2; Table 2). One further point that should not be overlooked is that seagrasses, and many submerged freshwater flowering plants, have photosynthesis almost entirely confined to the epidermis (Larkum et al. 2017). How this anatomical feature affects photosynthesis has been little explored, but its presence in a group of fairly diverse organisms that span several families suggests that it may be important and may affect photosynthesis. Therefore before accepting that seagrasses possess a C<sub>4</sub> metabolism, other explanations should be sought and in this search, gene expression is an important tool.

### Gene expression

Within the transcriptome of *Z. muelleri*, we discovered the presence of two different isoforms of PEPC; PEPC-1 and PEPC-2. Molecular differences coupled with differences in phylogenetic relations and gene structure between the two isoforms (Sánchez and Cejudo 2003) have suggested functional differences between the different isoforms in terrestrial plants. However the operation of these isoforms remains to be explored in marine angiosperms. When *Z. muelleri* plants were exposed to low O<sub>2</sub> conditions, we found a significant 2.2-fold decrease in PEPC-1 gene expression (Figure 3). In the terrestrial plant *Arabidopsis*, suppression of an isoform of PEPC via artificial microRNA (amiRNA) impaired root elongation and improved salt tolerance via increasing total PEPC activity (Wang et al.

2012). Little is known about the functionality of this isoform, while we recommend examining the effect of down regulation of PEPC-1 in *Z. muelleri*, the technique for genetic manipulation has not been established in *Z. muelleri* so far. In addition, although there was no statistical difference found in the expression of PEPC-2 in this study (Figure 3); there was a decline in response to low O<sub>2</sub> conditions. As mentioned in the introduction, although PEPC activity has been widely invoked as evidence of C<sub>4</sub> metabolism in aquatic autotrophs, it is also used to feed anaplerotic pathways that produce essential growth compounds such as amino acids (Aubry et



al. 2011). Therefore, it is possible that the down-regulation of PEPC under low O<sub>2</sub> reflects a decreased rate of Krebs cycle throughput however, as we only investigated the expression levels of PEPC as opposed to the activity level of the enzyme in response to low O<sub>2</sub>, it is important that the implications of these results are not overly extrapolated. Overall, our results indicate that, when exposed to low water-column O<sub>2</sub>, *Z. muelleri* plants (i) increase their photosynthetic activity, a characteristic of C<sub>3</sub> plant photosynthesis and (ii) down-regulate genes coding for PEPC, suggesting that these low O<sub>2</sub> conditions yield lower energy costs of photosynthesis.

HCO<sub>3</sub><sup>-</sup> can be utilised through extracellular dehydration via CA (Millhouse and Strother 1986a; Beer and Rehnberg 1997; Invers et al. 2001), although this alone does not constitute a CCM (Larkum et al. 2017). In the case of *Z. muelleri*, inhibition of CA activity via acetazolamide (a membrane-impermeant CA inhibitor, so only inhibiting extracellular CA) has been shown to inhibit photosynthetic use of HCO<sub>3</sub><sup>-</sup> (Millhouse and Strother 1986b; Koch et al. 2013; Borum et al. 2016). Of the 5 known independently evolved classes of CA ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\zeta$ ; Tripp et al. 2001) and the recently described  $\eta$  class (Del Prete et al. 2014), we investigated the expression levels of  $\gamma$ -CA. For this,  $\gamma$ -CA was selected as the sub-complexes are contained in the respiratory complex 1 (NADH:ubiquinone oxidoreductase) of plants and algae and in the mitochondrial respiratory electron transport chain, with sub-complexes serving as the entry point of electrons, potentially playing a role in photorespiration, probably as a HCO<sub>3</sub><sup>-</sup> transporter rather than as a normal CA (Braun and Zabaleta 2007; Martin et al. 2009). In agreement with photosynthetic response and regulation in PEPC genes, the significant down regulation of  $\gamma$ -CA genes (2.8-fold decrease: Figure 3) observed in *Z. muelleri* plants exposed to low O<sub>2</sub> suggests that this enzyme might be more critical for photosynthesis under ambient O<sub>2</sub> levels (i.e. lower DIC:O<sub>2</sub>) than under low O<sub>2</sub> levels.

In the freshwater aquatic monocot *Hydrilla verticillata*, C<sub>4</sub>-type photosynthesis is induced in C<sub>3</sub>-type photosynthesising leaves under warmer temperatures, limited CO<sub>2</sub>, increased O<sub>2</sub> and high photon irradiances (Bowes and Salvucci 1989). In this way, aquatic plants could have the capacity to acclimate to a changing climate, therefore highlighting the need to better understand these mechanisms especially in keystone seagrass species such as *Z. muelleri*.

Experimental manipulation of photosynthesis in aquatic organism can be complex as several factors need to be carefully considered. Firstly, avoiding pseudo-replication at the chamber/aquarium level is certainly desirable. In this respect, we recognize the limitations of our experimental design as each of the treatments (i.e. control and low O<sub>2</sub>) had one sump (100L plastic bin) feeding plastic container replicates, which is not ideal for full replication. However, we have used 4 container replicates for each treatment and we have randomized our sampling within each treatment to ameliorate some of the risks (Hurlbert 1984) and to separate the two O<sub>2</sub> levels from other potential effects originating from container replicates location on the table. This type of design is commonly used in experiments simulating ocean acidification (Sinutok et al. 2011; Sinutok et al. 2012; Sinutok et al. 2014). Secondly, it is also important to make sure that the experimental procedure does not affect multiple components of water chemistry, particularly inorganic carbon levels within different treatment tanks. While we did not perform any alkalinity measurements during the experiment, we can be confident that the various forms of inorganic carbon were in equilibrium during our experiment for the following reasons:

- i) The O<sub>2</sub> level in both the treatment and control tanks was stable throughout the experimental period (Supplementary Figure S2).
- ii) 99.9% pure CO<sub>2</sub> gas was used in the treatment tank to control the pH which was done via a pH controller connected to a pH/CO<sub>2</sub> controller (7074/2, TUNZE Aquarientechnik

GmbH, Germany), to maintain the same pH of 8.16 in both tanks (Supplementary Figure S3). This calibrated pH probe constantly measures the pH of the aquaria whereupon as pH starts to increase due to the flushing of nitrogen gas displacing the dissolved CO<sub>2</sub>, subsequently reducing the concentration of HCO<sub>3</sub><sup>-</sup>, the controller immediately switches on the CO<sub>2</sub> gas to stop the deviation and return the pH back to the experimental level (display accuracy of +/- 0.01 pH). Since this process occurs continuously, the pH deviates between 0.01-0.05 throughout the experimental period and these deviations were rectified automatically over several seconds.

iii) The tanks were kept in a temperature controlled room and salinity was kept constant throughout the experimental period.

iv) The time required for the various forms of inorganic carbon to reach equilibrium in seawater is at most 10s (see Zeebe and Wolf-Gladrow 2001).

This is the first study to combine microsensors and gene expression analyses to investigate responses to low O<sub>2</sub> in *Z. muelleri* and further studies with more sophisticated experimental set up are clearly needed to give more informative results.

## Conclusion

Ambiguous metabolic properties such as the ability to use HCO<sub>3</sub><sup>-</sup> and a C<sub>4</sub>-type photosynthetic quantum efficiency have led to some seagrass species being classified as C<sub>3</sub>C<sub>4</sub> intermediate plants (Beer et al. 1980; Beer and Wetzel 1981; Bowes and Salvucci 1989).

We suggest that the photosynthetic classification of *Z. muelleri* should also be carefully considered, as our results indicate that (i) ambient levels of O<sub>2</sub> affect the photophysiology of this seagrass, a characteristic of C<sub>3</sub> plants, and (ii) low O<sub>2</sub> levels induce the down-regulation of PEPC and P-CA genes. While regulation of these genes might not be strictly associated with a C<sub>4</sub> biochemistry, our data suggests that when the conditions are favourable for the

carboxylation reaction of RuBisCO, *Z. muelleri* down-regulates its CCM(s), thus altering its photophysiology. Future work involving the precise measurement of photorespiration and respiration is needed to show how photorespiration and respiration affect the photosynthetic response of seagrass to low O<sub>2</sub>. Furthermore, localization and activity of PEPCs,  $\gamma$ -CA and Rubisco and measurements of short-term (2-5 seconds) inorganic <sup>14</sup>C labelling products, is needed to show some type of compartmentalization between initial HCO<sub>3</sub><sup>-</sup> incorporation via PEPC and the final fixation of CO<sub>2</sub> via Rubisco, thereby supporting or not the role these enzymes play in the physiology of seagrasses. This research is not only needed to enable development of testable hypotheses to better direct future research, but also to improve the management and protection of these environmentally important marine angiosperms.

## References

Andersen CL, Jensen JL, Ørntoft TF (2004) Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64:5245-5250 doi:10.1158/0008-5472.can-04-0496

Anderson M, Gorley RN, Clarke RK (2008) Permanova+ for Primer: Guide to Software and Statistical Methods. PRIMER-E, Plymouth, UK

Aubry S, Brown NJ, Hibberd JM (2011) The role of proteins in C<sub>3</sub> plants prior to their recruitment into the C<sub>4</sub> pathway. *J Exp Bot* 62:3049-3059 doi:10.1093/jxb/err012

Baker NR (2008) Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo. *Annu Rev Plant Biol* 59:89-113 doi:10.1146/annurev.arplant.59.032607.092759

Beardall J, Quigg A, Raven JA (2003) Oxygen consumption: photorespiration and chlororespiration. In: *Photosynthesis in algae*. Springer, pp 157-181

Beck MW et al. (2001) The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates: a better understanding of the habitats that serve as

nurseries for marine species and the factors that create site-specific variability in nursery quality will improve conservation and management of these areas. *Bioscience* 51:633-641

Beer S, Björk M, Hellblom F, Axelsson L (2002) Inorganic carbon utilization in marine angiosperms (seagrasses). *Funct Plant Biol* 29:349-354

Beer S, Eshel A, Waisel Y (1977) Carbon metabolism in seagrasses I. The utilization of exogenous inorganic carbon species in photosynthesis. *J Exp Bot* 28:1180-1189

Beer S, Larsson C, Poryan O, Axelsson L (2000) Photosynthetic rates of *Ulva* (Chlorophyta) measured by pulse amplitude modulated (PAM) fluorometry. *Eur J Phycol* 35:69-74 doi:10.1080/09670260010001735641

Beer S, Rehnberg J (1997) The acquisition of inorganic carbon by the seagrass *Zostera marina*. *Aquat Bot* 56:277-283

Beer S, Shomer-Ilan A, Waisel Y (1980) Carbon Metabolism in Seagrasses II. Patterns of photosynthetic CO<sub>2</sub> incorporation. *J Exp Bot* 31:1019-1026

Beer S, Vilenkin B, Weil A, Veste M, Susel L, Eshel A (1998) Measuring photosynthetic rates in seagrasses by pulse amplitude modulated (PAM) fluorometry. *Mar Ecol Prog Ser* 174:293-300

Beer S, Wetzel RG (1981) Photosynthetic carbon metabolism in the submerged aquatic angiosperm *Scirpus subterminalis*. *Plant Sci Lett* 21:199-207

Benedict CR, Scott JR (1976) Photosynthetic carbon metabolism of a marine grass. *Plant Physiol* 57:876-880

Björk M, Haglund K, Ramazanov Z, Pedersén M (1993) Inducible mechanisms for HCO<sub>3</sub><sup>-</sup> utilization and repression of photorespiration in protoplasts and thalli of three species of *Ulva* (Chlorophyta). *J Phycol* 29:166-173 doi:10.1111/j.0022-3646.1993.00166.x

Björk M, Weil A, Semesi S, Beer S (1997) Photosynthetic utilisation of inorganic carbon by seagrasses from Zanzibar, East Africa. *Mar Biol* 129:363-366

Black C, Burris J, Everson R (1976) Influence of oxygen concentration on photosynthesis in marine plants. *Funct Plant Biol* 3:81-86 doi: { HYPERLINK

"http://dx.doi.org/10.1071/PP9760081" \h  HYPERLINK  
"http://dx.doi.org/10.1071/PP9760081" \h }

Blandon A, Zu Ermgassen PS (2014) Quantitative estimate of commercial fish enhancement by seagrass habitat in southern Australia. *Estuar Coast Shelf Sci* 141:1-8

Borum J, Pedersen O, Greve TM, Frankovich TA, Zieman JC, Fourqurean JW, Madden CJ (2005) The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, *Thalassia testudinum*. *J Ecol* 93:148-158  
doi:10.1111/j.13652745.2004.00943.x

Borum J, Pedersen O, Kotula L, Fraser MW, Statton J, Colmer TD, Kendrick GA (2016) Photosynthetic response to globally increasing CO<sub>2</sub> of co-occurring temperate seagrass species. *Plant, Cell Environ* 39:1240-1250 doi:10.1111/pce.12658

Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submersed aquatic macrophytes. *Aquat Bot* 34:233-266

Braun HP, Zabaleta E (2007) Carbonic anhydrase subunits of the mitochondrial NADH dehydrogenase complex (complex I) in plants. *Physiol Plant* 129:114-122

Brodersen KE et al. (2017) Sediment resuspension and deposition on seagrass leaves impedes internal plant aeration and promotes phytotoxic H<sub>2</sub>S intrusion. *Frontiers in Plant Science* 8  
doi:10.3389/fpls.2017.00657

Brodersen KE, Lichtenberg M, Paz L-C, Kühl M (2015) Epiphyte-cover on seagrass (*Zostera marina* L.) leaves impedes plant performance and radial O<sub>2</sub> loss from the below-ground tissue. *Front Mar Sci* 2:58

Brodersen KE, Nielsen DA, Ralph PJ, Kühl M (2014) A split flow chamber with artificial sediment to examine the below-ground microenvironment of aquatic macrophytes. *Mar Biol* 161:2921-2930 doi:10.1007/s00227-014-2542-3

Buapet P, Björk M (2016) The role of O<sub>2</sub> as an electron acceptor alternative to CO<sub>2</sub> in photosynthesis of the common marine angiosperm *Zostera marina* L. *Photosynth Res*:1-11

- Buapet P, Rasmusson LM, Gullström M, Björk M (2013) Photorespiration and carbon limitation determine productivity in temperate seagrasses. *PloS one* 8:e83804
- Bulmer R, Kelly S, Jeffs A (2016) Light requirements of the seagrass, *Zostera muelleri*, determined by observations at the maximum depth limit in a temperate estuary, New Zealand. *N Z J Mar Freshwat Res*:1-12
- Bustin SA et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611-622
- Carlson P, Yarbrow L, Sargent W, Arnold H (1988) Hypoxic stress in *Thalassia testudinum*: Evidence from diurnal changes in rhizome gas composition. *Eos* 69:733-746
- Chartrand KM, Bryant CV, Carter AB, Ralph PJ, Rasheed MA (2016) Light thresholds to prevent dredging impacts on the Great Barrier Reef seagrass, *Zostera muelleri* ssp. *capricorni*. *Front Mar Sci* 3:106
- Chi S, Wu S, Yu J, Wang X, Tang X, Liu T (2014) Phylogeny of C<sub>4</sub>-photosynthesis enzymes based on algal transcriptomic and genomic data supports an archaeal/proteobacterial origin and multiple duplication for most C<sub>4</sub>-related genes. *PloS one* 9:e110154
- Chollet R, Vidal J, O'Leary MH (1996) Phosphoenol pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annu Rev Plant Biol* 47:273-298
- Colman B, Norman EG (1997) Serine synthesis in cyanobacteria by a nonphotorespiratory pathway. *Physiol Plant* 100:133-136
- Costanza R et al. (1997) The value of the world's ecosystem services and natural capital. *Nature* 387:253-260
- Del Prete S, Vullo D, Fisher GM, Andrews KT, Poulsen S-A, Capasso C, Supuran CT (2014) Discovery of a new family of carbonic anhydrases in the malaria pathogen *Plasmodium falciparum*—The η-carbonic anhydrases. *Bioorg Med Chem Lett* 24:4389-4396
- Downton W, Bishop D, Larkum AWD, Osmond C (1976) Oxygen inhibition of photosynthetic oxygen evolution in marine plants. *Funct Plant Biol* 3:73-79

Drechsler Z, Beer S (1991) Utilization of inorganic carbon by *Ulva lactuca*. Plant Physiol 97:1439-1444

Figuerola FL, Jerez CG, Korbee N (2013) Use of in vivo chlorophyll fluorescence to estimate photosynthetic activity and biomass productivity in microalgae grown in different culture systems. Lat Am J Aquat Res 41:801-819 doi:103856/vol41-issue5-fulltext-1

Fourqurean JW et al. (2012) Seagrass ecosystems as a globally significant carbon stock. Nature Geoscience 5:505-509

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances “blue carbon” sequestration in coastal waters. PloS one 8:e72469

Greve TM, Borum J, Pedersen O (2003) Meristematic oxygen variability in eelgrass (*Zostera marina*). Limnol Oceanogr 48:210-216 doi:10.4319/lo.2003.48.1.0210

Harlin MM (1995) Changes in major plant groups following nutrient enrichment. Eutrophic Shallow Estuaries and Lagoons CRC Press, Inc, Boca Raton, Florida:173-187

Hellblom F, Axelsson L (2003) External  $\text{HCO}_3^-$  dehydration maintained by acid zones in the plasma membrane is an important component of the photosynthetic carbon uptake in *Ruppia cirrhosa*. Photosynth Res 77:173-181

Hellblom F, Beer S, Björk M, Axelsson L (2001) A buffer sensitive inorganic carbon utilisation system in *Zostera marina*. Aquat Bot 69:55-62

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54:187-211

Invers O, Zimmerman RC, Alberte RS, Perez M, Romero J (2001) Inorganic carbon sources for seagrass photosynthesis: an experimental evaluation of bicarbonate use in species inhabiting temperate waters. J Exp Mar Biol Ecol 265:203-217

Jiménez C, Niell FX, Algarra P (1987) Photosynthetic adaptation of *Zostera noltii* Hornem. Aquat Bot 29:217-226

Jørgensen BB, Revsbech NP (1985) Diffusive boundary layers and the oxygen uptake of sediments and detritus. Limnol Oceanogr 30:111-122 doi:10.4319/lo.1985.30.1.0111



Koch M, Bowes G, Ross C, Zhang XH (2013) Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Global Change Biol* 19:103-132

Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23:1289-1291

Kühl M, Cohen Y, Dalsgaard T, Jørgensen B, Revsbech NP (1995) The microenvironment and photosynthesis of *zooxanthellae* in scleractinian corals studied with microsensors for O<sub>2</sub>, pH and light. *Mar Ecol Prog Ser* 117:159-172

Larkum A, Davey P, Kuo J, Ralph P, Raven J (2017) Carbon-concentrating mechanisms in seagrasses. *J Exp Bot* in press doi:10.1093/jxb/erx206

Larkum A, den Hartog C (1989) Evolution and biogeography of seagrasses. In: Larkum AWD, McComb AJ, Shepherd SA (eds) *Biology of Seagrasses*. 1 edn. Elsevier Pub Co, Amsterdam, The Netherlands, pp 112-156

Larsson C, Lennart A, Ryberg H, Beer S (1997) Photosynthetic carbon utilization by *Enteromorpha intestinalis* (Chlorophyta) from a Swedish rockpool. *Eur J Phycol* 32:49-54 doi:10.1080/09541449710001719365

Lee H et al. (2016) The genome of a southern hemisphere seagrass species (*Zostera muelleri*). *Plant Physiol*:00868.02016

Les DH, Cleland MA, Waycott M (1997) Phylogenetic studies in Alismatidae, II: evolution of marine angiosperms (seagrasses) and hydrophily. *Syst Bot*:443-463

Lichtenberg M, Kühl M (2015) Pronounced gradients of light, photosynthesis and O<sub>2</sub> consumption in the tissue of the brown alga *Fucus serratus*. *New Phytol* 207:559-569 doi:10.1111/nph.13396

Long MH, McGlathery KJ, Zieman JC, Berg P (2008) The role of organic acid exudates in liberating phosphorus from seagrass-vegetated carbonate sediments. *Limnol Oceanogr* 53:2616-2626 doi:10.4319/lo.2008.53.6.2616

Martin V et al. (2009) Recombinant plant gamma carbonic anhydrase homotrimers bind inorganic carbon. *FEBS Lett* 583:3425-3430 doi:10.1016/j.febslet.2009.09.055

Mass T, Genin A, Shavit U, Grinstein M, Tchernov D (2010) Flow enhances photosynthesis in marine benthic autotrophs by increasing the efflux of oxygen from the organism to the water. *Proceedings of the National Academy of Sciences* 107:2527-2531

McLeod E et al. (2011) A blueprint for blue carbon: toward an improved understanding of the role of vegetated coastal habitats in sequestering CO<sub>2</sub>. *Front Ecol Environ* 9:552-560  
doi:10.1890/110004

Millhouse J, Strother S (1986a) The effect of pH on the inorganic carbon source for photosynthesis in the seagrass *Zostera muelleri* Irmisch ex Aschers. *Aquat Bot* 24:199-209

Millhouse J, Strother S (1986b) Salt-stimulated bicarbonate-dependent photosynthesis in the marine angiosperm *Zostera muelleri*. *J Exp Bot* 37:965-976

Olsen JL et al. (2016) The genome of the seagrass *Zostera marina* reveals angiosperm adaptation to the sea. *Nature* 530:331-335

Papenbrock J (2012) Highlights in seagrasses' phylogeny, physiology, and metabolism: what makes them special? *ISRN Botany* 2012 doi:10.5402/2012/103892

Pedersen O, Colmer TD, Borum J, Zavala-Perez A, Kendrick GA (2016) Heat stress of two tropical seagrass species during low tides – impact on underwater net photosynthesis, dark respiration and diel in situ internal aeration. *New Phytol* 210:1207-1218  
doi:10.1111/nph.13900

Penhale PA, Wetzel RG (1983) Structural and functional adaptations of eelgrass (*Zostera marina* L.) to the anaerobic sediment environment. *Can J Bot* 61:1421-1428

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45-e45

Pierrot D, Lewis E, Wallace D (2006) MS Excel program developed for CO<sub>2</sub> system calculations: ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee

Pregnall A, Smith R, Kursar TA, Alberte R (1984) Metabolic adaptation of *Zostera marina* (eelgrass) to diurnal periods of root anoxia. *Mar Biol* 83:141-147

Procaccini G, Olsen JL, Reusch TB (2007) Contribution of genetics and genomics to seagrass biology and conservation. *J Exp Mar Biol Ecol* 350:234-259

Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 313:856-862

Ralph P, Durako MJ, Enriquez S, Collier C, Doblin M (2007) Impact of light limitation on seagrasses. *J Exp Mar Biol Ecol* 350:176-193

Raven JA (1984) *Energetics and transport in aquatic plants*. A.R. Liss, New York, USA

Raven JA (2014) Speedy small stomata? *J Exp Bot* 65:1415-1424 doi:10.1093/jxb/eru032

Raven JA, Beardall J (2016) The ins and outs of CO<sub>2</sub>. *J Exp Bot* 67:1-13 doi:10.1093/jxb/erv451

Raven JA, Farquhar GD (1990) The influence of N metabolism and organic acid synthesis on the natural abundance of isotopes of carbon in plants. *New Phytol* 116:505-529

Roberts DG, Moriarty DJW (1987) Lacunal gas discharge as a measure of productivity in the seagrasses *Zostera capricorni*, *Cymodocea serrulata* and *Syringodium isoetifolium*. *Aquat Bot* 28:143-160 doi: { HYPERLINK "http://dx.doi.org/10.1016/0304-3770(87)90036-2" \h }  
HYPERLINK "http://dx.doi.org/10.1016/0304-3770(87)90036-2" \h } HYPERLINK  
"http://dx.doi.org/10.1016/0304-3770(87)90036-2" \h } HYPERLINK  
"http://dx.doi.org/10.1016/0304-3770(87)90036-2" \h } HYPERLINK  
"http://dx.doi.org/10.1016/0304-3770(87)90036-2" \h } HYPERLINK  
"http://dx.doi.org/10.1016/0304-3770(87)90036-2" \h }

Sánchez R, Cejudo FJ (2003) Identification and expression analysis of a gene encoding a bacterial-type phosphoenolpyruvate carboxylase from *Arabidopsis* and rice. *Plant Physiol* 132:949-957

Schliep M, Pernice M, Sinutok S, Bryant C, York P, Rasheed M, Ralph P (2015) Evaluation of reference genes for RT-qPCR studies in the seagrass *Zostera muelleri* exposed to light limitation. *Sci Rep* 5:17051 doi:10.1038/srep17051

Schwarz AM (2004) Contribution of photosynthetic gains during tidal emersion to production of *Zostera capricorni* in a North Island, New Zealand estuary. *N Z J Mar Freshwat Res* 38:809-818

Short FT et al. (2011) Extinction risk assessment of the world's seagrass species. *Biol Conserv* 144:1961-1971 doi: { HYPERLINK "http://dx.doi.org/10.1016/j.biocon.2011.04.010" \h }  
HYPERLINK "http://dx.doi.org/10.1016/j.biocon.2011.04.010" \h }

Sinutok S, Hill R, Doblin MA, Kühl M, Ralph PJ (2012) Microenvironmental changes support evidence of photosynthesis and calcification inhibition in *Halimeda* under ocean acidification and warming. *Coral Reefs* 31:1201-1213 doi:10.1007/s00338-012-0952-6

Sinutok S, Hill R, Doblin MA, Wuhrer R, Ralph PJ (2011) Warmer more acidic conditions cause decreased productivity and calcification in subtropical coral reef sediment-dwelling calcifiers. *Limnol Oceanogr* 56:1200-1212 doi:10.4319/lo.2011.56.4.1200

Sinutok S, Hill R, Kühl M, Doblin MA, Ralph PJ (2014) Ocean acidification and warming alter photosynthesis and calcification of the symbiont-bearing foraminifera *Marginopora vertebralis*. *Mar Biol* 161:2143-2154 doi:10.1007/s00227-014-2494-7

Smith RD, Dennison WC, Alberte RS (1984) Role of seagrass photosynthesis in root aerobic processes. *Plant Physiol* 74:1055-1058

Spilling K, Titelman J, Greve TM, Kühl M (2010) Microsensor measurements of the external and internal microenvironment of *Fucus vesiculosus* (Phaeophyceae). *J Phycol* 46:1350-1355 doi:10.1111/j.1529-8817.2010.00894.x

Staehr PA, Borum J (2011) Seasonal acclimation in metabolism reduces light requirements of eelgrass (*Zostera marina*). *J Exp Mar Biol Ecol* 407:139-146

Tripp BC, Smith K, Ferry JG (2001) Carbonic anhydrase: new insights for an ancient enzyme. *J Biol Chem* 276:48615-48618 doi:10.1074/jbc.R100045200

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. *Nucleic Acids Res* 40:e115-e115

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:1

Vogel S (1994) *Life in moving fluids: the physical biology of flow*. Princeton University Press, New Jersey, USA

Voznesenskaya EV, Franceschi VR, Kierats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C<sub>4</sub> plant photosynthesis. *Nature* 414:543-546

Waghmode A, Joshi G (1983) Significance of phosphoglycollate phosphatase and 3-phosphoglycerate phosphatase in photosynthetic carbon assimilation in some marine plants (*Cerriops*, *Lumnitzera*, *Aegiceras*, *Aeluropus*, *Halophila*). *Photosynthetica* 17:193-197

Wang F, Liu R, Wu G, Lang C, Chen J, Shi C (2012) Specific downregulation of the bacterial-type PEPC gene by artificial microRNA improves salt tolerance in *Arabidopsis*. *Plant Mol Biol Report* 30:1080-1087

Webb WL, Newton M, Starr D (1974) Carbon dioxide exchange of *Alnus rubra*. *Oecologia* 17:281-291

Zeebe RE, Wolf-Gladrow DA (2001) *CO<sub>2</sub> in seawater: equilibrium, kinetics, isotopes*. vol 65. Gulf Professional Publishing,

Zimmerman RC, Kohrs DG, Steller DL, Alberte RS (1997) Impacts of CO<sub>2</sub> enrichment on productivity and light requirements of eelgrass. *Plant Physiol* 115:599-607

857 **Table 1.** Reference genes and target genes investigated in *Zostera muelleri* by using RT-qPCR. Accession numbers of the closest sequence  
858 matches available online in the data repository for *Zostera marina* EST ([HYPERLINK "http://drzompo.uni-muenster.de/"](http://drzompo.uni-muenster.de/) \h [HYPERLINK](http://drzompo.uni-muenster.de/)  
859 ["http://drzompo.uni-muenster.de/"](http://drzompo.uni-muenster.de/) \h [HYPERLINK "http://drzompo.uni-muenster.de/"](http://drzompo.uni-muenster.de/) \h [HYPERLINK "http://drzompo.uni-muenster.de/"](http://drzompo.uni-muenster.de/)  
860 \h } primers sequences, amplicon length, melting temperature, geometric mean of cycle threshold (CT) and RT-qPCR efficiency are indicated.  
861 GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; EloF: Translation initiation factor 1 subunit beta; Calmodulin; TubB: Tubulin beta-1  
862 chain; Actin; PolyA: Poly(A) RNA polymerase; S4: 30S ribosomal protein S4; PEPC-1: Phosphoenolpyruvate carboxylase – isoform 1; PEPC-2:  
863 Phosphoenolpyruvate carboxylase – isoform 2 and  $\gamma$ -CA:  $\gamma$  Carbonic anhydrase.

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Name	Accession	Forward primer	Reverse primer	Length	$T_m$	$C_T$	Efficiency	number	(bp)	(%)
GAPDH	Zoma_C_c6252	CGGTTACTGTAGCCCCACTC	CAAAGGCTGGGATTGGTTTA	79	59.9	25	88			
EloF	Zoma_C_c59090	AAGCAAAGGCGTCACTTGAT	TCTGCTGCCTTCTTCTCCTC	82	59.9	24	104			
Calmodulin	Zoma_B_i07192	ATCCATCCTGGTCTTTGTCG	CACTGTGATCCACTCGTTGG	197	60.1	23	114			
TubB	Zoma_Contig120	GGACAAATCTTCCGTCCAGA	TCCAGATCCAGTTCCACCTC	195	60	24	88			
Actin	Zoma_ZMF02257	TAAGGTCGTTGCTCCTCCTG	ACTCTGCCTTTGCAATCCAC	104	60.4	26	110			
PolyA	Zoma_C_c36619	GCTGCTCGTTCAAATTCCTC	ATGACCGCCATTTAATCTGC	112	59.9	29	93			
S4	Zoma_Contig219	ATGGTCTGACAGAGCGACAA	TGTTATCCAAACGCATCTCG	108	59.7	29	114			
PEPC-1	KMZ56135	AGGCAAATTCGGACTTCCT	GAGGACGCAGTGTGACAGA	84	60.1	29	99			
PEPC-2	KMZ58048	TGGCTGTTGTAGCCACTGAG	TCTGTCTCTGGTGTGGCAAG	91	60	28	97			
$\gamma$ -CA	KMZ56166	AGGTCATGGTGTGCTGCCTTC	CAGCAACCATTCCGTTCTTT	110	60.1	28	104			

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868 **Table 2.** The initial slope of the P-I curve in the light-limiting phase ( $\alpha$ ), maximum net photosynthetic rate ( $P_{max}$ ), dark respiration rate (R),  
869 compensation irradiance ( $E_c$ ) and minimum saturating irradiance ( $E_k$ ) in *Zostera muelleri* leaves exposed to water-column  $O_2$  levels of ~231

870  $\mu\text{mol O}_2 \text{ L}^{-1}$  (control) and  $\sim 8 \mu\text{mol O}_2 \text{ L}^{-1}$  (low  $\text{O}_2$ ). Values are given as a mean  $\pm$ SEM (n=3); with their corresponding P values (Permutation  
 871 ttest), where \* indicate significant difference between treatments on a 5% level.

	$\alpha$	$P_{\text{max}}$ (nmol $\text{O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ )	$R$ (nmol $\text{O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ )	$E_k$ ( $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ )	$E_c$ ( $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ )
<b>Control <math>\text{O}_2</math></b>	10.95 $\pm$ 0.4	668.14 $\pm$ 80.3	-117.09 $\pm$ 12.5	60.82 $\pm$ 6.0	5.19 $\pm$ 0.8
<b>Low <math>\text{O}_2</math></b>	13.34 $\pm$ 0.1	687.85 $\pm$ 49.3	-21.42 $\pm$ 3.9	51.66 $\pm$ 4.2	0.71 $\pm$ 0.1
<b><i>P</i></b>	0.0064*	0.7974	0.0018*	0.2892	0.0012*

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874 Figure Legends

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876 **Fig. 1** Vertical O<sub>2</sub> concentration micro-profiles measured at the surface of *Zostera muelleri*  
877 leaves. Filled circles and solid lines represent data from the control leaves (i.e. leaves exposed  
878 to a water-column O<sub>2</sub> concentration of ~231 μmol O<sub>2</sub> L<sup>-1</sup>), while open circles and dashed  
879 lines represent data from leaves exposed to low O<sub>2</sub> conditions (i.e. ~8 μmol O<sub>2</sub> L<sup>-1</sup>). Incident  
880 photon irradiances are indicated by the figure legend (i.e. 0, 25, 50, 100, 200, 500 and 700  
881 μmol photons m<sup>-2</sup> s<sup>-1</sup>). Error bars are ± standard error of the mean (SEM). Y = 0 indicate the  
882 leaf tissue surface. n=3

883 **Fig. 2** Net photosynthesis of *Zostera muelleri* leaves at an incident photon irradiance of 0, 25,  
884 50, 100, 200, 500 and 700 μmol photons m<sup>-2</sup> s<sup>-1</sup>. Data points were fitted with an exponential  
885 saturation function (Webb et al., 1974) with an added respiration term, R, to account for  
886 respiration (Spilling et al., 2010). Black squares and line represent data of leaves kept in ~231  
887 μmol O<sub>2</sub> L<sup>-1</sup> (i.e. control plants), while open triangles and dashed line represent data of leaves  
888 kept in ~8 μmol O<sub>2</sub> L<sup>-1</sup> (i.e. low O<sub>2</sub> treatment). Error bars are ±SEM; while statistically  
889 different values are indicated by \* (Permutation t-test, P< 0.05). n=3



892 **Fig. 3** Normalized relative quantity (NRQ) of Phosphoenolpyruvate carboxylase (PEPC-1  
893 isoform 1 and PEPC-2 isoform 2) and  $\gamma$  Carbonic anhydrase ( $\gamma$ -CA) in *Zostera muelleri* under  
894 control (solid bars) and low O<sub>2</sub> conditions (open bars) relative to the two most stable  
895 reference genes: S4 and GADPH. Statistical differences in the mean are indicated with \*  
896 (Permutation t-test, P < 0.05) and error bars are  $\pm$ SEM. n=4

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